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The biosynthesis of 3-hydroxypropylmercapturic acid from cyclophosphamide

P. M. GILES†

Department of Biochemistry, St Thomas's Hospital Medical School, London, U.K.

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1. 3-Hydroxypropylmercapturic acid has been identified in the urine of rats dosed with cyclophosphamide, isophosphamide, and trilophosphamide and was isolated as its dicyclohexylammonium salt from the urine of rats dosed with cyclophosphamide.

2. Rats excreted in their urine 55·5% of the ^{14}C of an i.p. dose (200 mg/kg) of [4-^{14}C]cyclophosphamide during the first 24 h after administration, and a further 6·6% during the subsequent 24 h. Of the total radioactivity excreted during the first 24 h, unchanged cyclophosphamide represented 19·3% or less, 41·6% was due to the major metabolite, carboxyphosphamide, and 3-hydroxypropylmercapturic acid represented 11·9%. The ^{14}C label in the mercapturic acid was located in the *S*-substituent.

3. 3-Hydroxypropylmercapturic acid was the only sulphur-containing metabolite of cyclophosphamide found in the blood and liver of rats, but 3-hydroxy[^{14}C]propylmercapturic acid and *S*-(3-hydroxy[^{14}C]propyl)-L-cysteine were tentatively identified in the bile of a rat dosed with [^{14}C]cyclophosphamide (120 mg/kg; i.p.).

4. *S*-(3-Hydroxypropyl)-L-cysteine and 3-hydroxypropylmercapturic acid were detected in the liver of rats dosed with allyl alcohol.

5. Acrolein undergoes a complex reaction with GSH *in vitro* producing a number of products, the major one of which, after reduction with borohydride, is chromatographically identical to *S*-(3-hydroxypropyl)glutathione. The reduced product, *S*-(3-hydroxypropyl)glutathione and *S*-(3-hydroxypropyl)-L-cysteine are all metabolized by the rat into 3-hydroxypropylmercapturic acid and two minor metabolites.

6. 3-Hydroxypropylmercapturic acid is converted by the rat into 2-carboxyethylmercapturic acid and one other metabolite. Neither of these metabolites was found in the urine of rats dosed with cyclophosphamide.

7. A possible pathway for the formation of 3-hydroxypropylmercapturic acid from cyclophosphamide is discussed.

Introduction

Cyclophosphamide (figure 1) is a drug (Arnold and Bourseaux 1958) widely used in the treatment of cancer (Hill 1975). Therapeutically, cyclophosphamide itself is inactive, but *in vivo* it is converted, mainly by the liver, into alkylating, cytotoxic and other metabolites (for reviews see Torkelson *et al.* 1974, Hill 1975).

The general consensus of opinion is that the drug is initially converted (figure 1) into 4-hydroxycyclophosphamide by liver microsomal mixed-function oxidases using molecular oxygen and NADPH (Hill, Laster and Struck 1972, Connors *et al.* 1974a, b). This metabolite then equilibrates with its acyclic tautomer, aldophosphamide, which subsequently undergoes either spontaneous β-elimination yielding acrolein and phosphoramide mustard (Connors *et al.* 1974a, b) or oxidation, catalysed by aldehyde dehydrogenase, forming carboxyphosphamide (Cox, Phillips and Thomas 1975). The relative amounts of acrolein, phosphoramide mustard and carboxyphosphamide formed from aldophosphamide in a tissue depends on the activity of aldehyde dehydrogenase (Cox *et al.* 1975). Phosphoramide mustard

† Present address: Department of Child Health, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK.

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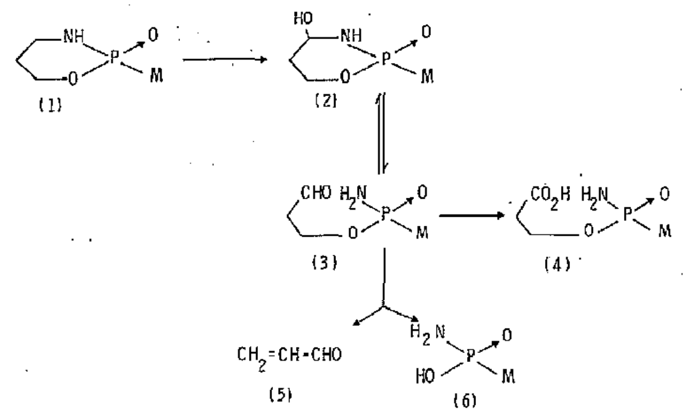


Figure 1. The metabolic conversion of cyclophosphamide into acrolein and phosphoramide mustard. (1), Cyclophosphamide; (2), 4-Hydroxycyclophosphamide; (3), Aldophosphamide; (4), Carboxyphosphamide; (5), Acrolein; (6), Phosphoramide mustard; M, Mustard group $-N(CH_2CH_2Cl)_2$.

(Connors *et al.* 1974 a, Colvin *et al.* 1976) and acrolein (Alarcon, Meienhofer and Atherton 1972) are cytotoxic, unlike carboxyphosphamide (Struck *et al.* 1971), and are currently thought to be responsible for the therapeutic effect of cyclophosphamide *in vivo*.

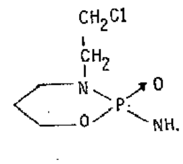
Tentative evidence has previously been obtained for the excretion of 3-hydroxypropylmercapturic acid in the urine of rats and humans after administration of cyclophosphamide (Kaye and Young 1974, Alarcon 1976) and it has been suggested that the drug might be converted into the mercapturic acid via the intermediate formation of acrolein (Kaye and Young 1974). This suggestion was based on the identification of acrolein as a liver microsomal metabolite of cyclophosphamide *in vitro* (Alarcon and Meienhofer 1971) and the observation that administration of acrolein to rats results in the excretion in the urine of 3-hydroxypropylmercapturic acid (Kaye 1971, 1973).

The formation of mercapturic acids *in vivo* is considered to take place by the mechanism proposed by Barnes, James and Wood (1959) and Bray, Franklin and James (1959). This involves the reaction of a mercapturic acid precursor with the –SH group of GSH yielding an *S*-substituted glutathione (glutathione conjugate) which is subsequently metabolized into a mercapturic acid via the intermediate formation of an *S*-substituted cysteine. Kaye (1971, 1973) suggested that this pathway could be in operation for the conversion of acrolein into 3-hydroxypropylmercapturic acid *in vivo*.

This paper reports the isolation of 3-hydroxypropylmercapturic acid from the urine of rats dosed with cyclophosphamide, its identification as a metabolite of [^{14}C]cyclophosphamide *in vivo* and some studies concerned with the mechanism of this route of metabolism in the rat.

Materials and methods

Cyclophosphamide (2-[bis-(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) (>89%) was obtained from Ward Blenkinsop Pharmaceuticals Ltd, Bracknell, Berks., and isophosphamide (>98%) and trilophosphamide (>98%) (figure 2) were gifts from this company. [4-^{14}C]Cyclophosphamide (sp. activity: 2·75 Ci/mol) was obtained from New England Nuclear Chemicals Ltd, Boston, USA, and was >99% pure by paper chromatography in solvents A, B and C, and by t.l.c. on silica gel in benzene–chloroform–acetic acid–methanol (40 : 40 : 10 : 10, by vol.).



Isophosphamide

Figure 2. The chemi

Carboxyphosphamide (prepare
M. Jarman) were donated by Dr
Hospital, London SW3.
Acrolein (99%) was purchase
method similar to that described
Dicyclohexylammonium 3-hy
and Kaye, Clapp and Young (1
hydroxypropylmercapturate and
synthesized by the method of Barn
two compounds (Kaye 1971, 1
chromatography of these syntheti
same compounds previously prep
al. (1972).
Allylmercapturic acid was a g
mercapturate and *S*-(3-hydroxyp
Trimethylsilyl derivatives of hy
anhydrous pyridine using TRI-S
and trimethylchlorosilane as rep
Sulphoxides were synthesize
Raney nickel was prepared fro
method of Vogel (1948).
Free mercapturic acids were
of the salt through a column of Zc
of the eluate was 5. The total elu
dried over P_2O_5 under vacuum.

Animals and dosing

Male albino rats of the CF
Hospital Medical School, Lond
all experiments. The animals w
continuous access to water.
Foreign compounds were a
region (s.c.), intraperitoneally (i
contrary: Cyclophosphamide–
trilophosphamide—i.p., 150 n
0·135 μCi/mg) i.p., 200 mg/kg i
cysteine and 3-hydroxypropyl
respectively; allyl alcohol and
animals received saline or arac
dosing. Rats treated with sodiu
at a level of 1 g/litre for six da
During experimental work
of urine from the faeces. The u
for a further 24 h after a one-
Where the rats had been do
was required, animals were pl
urine was collected in all-gla
apparatus was washed with wa
appropriate 24 h urine sample
under reduced pressure.

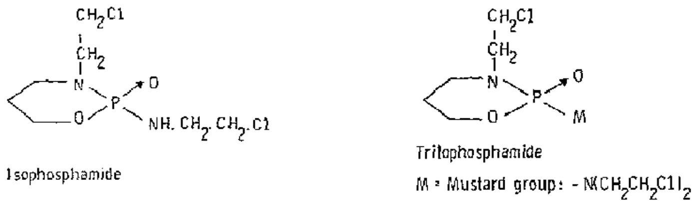


Figure 2. The chemical structures of isophosphamide and trilophosphamide.

Carboxyphosphamide (prepared by Mr L. Griggs) and 4-ketocyclophosphamide (synthesized by Dr M. Jarman) were donated by Dr P. Cox of the Chester Beatty Research Institute, Royal Marsden Hospital, London SW3.

Acrolein (99%) was purchased from Aldrich Chemicals Ltd, and was redistilled before use by a method similar to that described by Gillman (1943).

Dicyclohexylammonium 3-hydroxypropylmercapturate was prepared as described by Kaye (1971) and Kaye, Clapp and Young (1972). *S*-(3-Hydroxypropyl)-L-cysteine, dicyclohexylammonium 2-hydroxypropylmercapturate and dicyclohexylammonium 2-hydroxy-1-methylethylmercapturate were synthesized by the method of Barnsley (1966) without separation of the (+)- and (−)-isomers of the latter two compounds (Kaye 1971, 1973). The i.r. spectra, melting points and behaviour on paper chromatography of these synthetic compounds were identical to those of analytically pure samples of the same compounds previously prepared in this department and described by Kaye (1971, 1973) and Kaye *et al.* (1972).

Allylmercapturic acid was a gift from Dr C. M. Kaye. Bisdicyclohexylammonium 2-carboxyethylmercapturate and *S*-(3-hydroxypropyl)glutathione were prepared as previously described (Giles 1976). Trimethylsilyl derivatives of hydroxypropylmercapturic acids were prepared from the free acids in anhydrous pyridine using TRI-SIL (Pierce Chemicals, Rockford, Illinois, USA) or hexamethyldisilazane and trimethylchlorosilane as reported by Giles (1976).

Sulphoxides were synthesized according to the method of Kaye (1971) and Kaye *et al.* (1972).

Raney nickel was prepared from nickel-aluminium alloy (BDH Chemicals Ltd, Poole, Dorset) by the method of Vogel (1948).

Free mercapturic acids were prepared from their dicyclohexylammonium salts by passing an aq. soln. of the salt through a column of Zeo-Karb 225 (H^+ form). The column was washed with water until the pH of the eluate was 5. The total eluate was evaporated to dryness at 30°C under reduced pressure and then dried over P_2O_5 under vacuum.

Animals and dosing

Male albino rats of the CFE strain (supplied by the Herbert Research Laboratories, St Thomas's Hospital Medical School, London), body weight 180–220 g (except where otherwise stated) were used for all experiments. The animals were fed on rat cakes (Spillers Autoclaved Small Animal Diet) and had continuous access to water.

Foreign compounds were administered in 0·9% (w/v) NaCl either subcutaneously in the lumbar region (s.c.), intraperitoneally (i.p.) or by gavage (stomach tube), as follows except where described to the contrary: Cyclophosphamide—s.c., i.p., or gavage, 100–200 mg/kg; isophosphamide—i.p., 200 mg/kg; trilophosphamide—i.p., 150 mg/kg in arachis oil (1 ml); [^{14}C]cyclophosphamide (sp. activity 0·135 μCi/mg) i.p., 200 mg/kg in water (1 ml); *S*-(3-hydroxypropyl)glutathione, *S*-(3-hydroxypropyl)-L-cysteine and 3-hydroxypropylmercapturic acid—i.p., 228–343 mg/kg, 100–150 mg/kg and 280 mg/kg respectively; allyl alcohol and acrolein—s.c., 5% (v/v) soln. in arachis oil (1 ml). Experimental control animals received saline or arachis oil. All animals were lightly anaesthetized with diethyl ether before dosing. Rats treated with sodium phenobarbital had this compound introduced into their drinking water at a level of 1 g/litre for six days.

During experimental work the animals were housed in metabolism cages allowing separate collection of urine from the faeces. The urine was collected for 24 h after administration of test compounds, and then for a further 24 h after a one-hour feeding period.

Where the rats had been dosed with [^{14}C]cyclophosphamide, and quantitative collection of the urine was required, animals were placed in a metabolism cage divided by wire mesh to keep them apart, and the urine was collected in all-glass apparatus. After each 24 h period of urine collection, the collecting apparatus was washed with water and the washings were filtered through a glass sinter and mixed with the appropriate 24 h urine sample. The urine sample plus washings was then concentrated to 25 ml at 30°C under reduced pressure.

Paper chromatography

Whatman 3 MM papers (47 × 23 cm) were used except where otherwise stated. Papers used for preparative work were washed with the developing solvent and with water, but papers used for analytical work received no pretreatment. All chromatograms were developed by the descending technique at laboratory temperature with one of the following solvent mixtures: A, butan-1-ol–acetic acid–water (12 : 3 : 5, by vol.); B, butan-1-ol–pyridine–3 M ammonia (4 : 3 : 3, by vol.); C, propan-1-ol–water–ammonia soln. (sp. gr. 0·88) (80 : 19 : 1, by vol.); D, propan-1-ol–chloroform–methanol–3 M ammonia (50 : 30 : 10 : 10, by vol.); E, acetone–ethanol–ammonia soln. (sp. gr. 0·88) (8 : 1 : 1, by vol.).

Compounds purified by preparative paper chromatography were applied in aq. soln. as streaks (10 cm) to the bases of chromatography papers. After development, the appropriate area of each chromatogram (determined by reference markers at the edge of the paper) was eluted with water, and the eluate was either lyophilized or concentrated at 30°C under reduced pressure.

After development, the chromatograms were dried in a current of warm air. Sulphur-containing compounds were visualized by dipping the papers in the platinum reagent as described by Toennies and Kolb (1951) and modified by Barnsley, Thomson and Young (1964). Amino compounds were subsequently detected on the same chromatogram by dipping the papers in the ninhydrin reagent (0·2% ninhydrin in acetone–pyridine, 95 : 5, by vol.). Aldehydes were detected by dipping the chromatograms in dinitrophenylhydrazine reagent (Gray and Barnsley 1971), and *N*-acetylalanine was visualized with the alkaline phenol red reagent described by Buffa, Peters and Wakelin (1951). Chromatograms were dried in a current of warm air immediately after application of these reagents. Alkylating agents were detected by a modification of the method of Hill *et al.* (1972) which involved spraying the chromatograms with 1% (w/v) 4-(4-nitrobenzyl)pyridine in acetone followed by heating at 100°C for 15–20 minutes and then respraying with 3% (w/v) KOH in ethanol.

The R_F and R_X values of some reference compounds are given in table 1.

Table 1. Separation of synthetic compounds by paper chromatography.

Compound	R_F in solvent A	R_F in solvent B	R_F in solvent C	R_X in solvent D
3-Hydroxypropylmercapturic acid†	0·76	0·52	0·47	0·50 (0·31)
2-Hydroxypropylmercapturic acid†	0·78	0·53	0·48	0·54
2-Hydroxy-1-methylethylmercapturic acid†	0·80	0·56	0·51	0·63
S-(3-Hydroxypropyl)-L-cysteine‡	0·38	0·41	0·36	0·32 (0·20)
S-(3-Hydroxypropyl)glutathione‡	0·34	0·16	0·10	0·03
D,L-α-Alanine§	0·36	0·29	0·28	0·24
4-Ketocyclophosphamide¶	—	—	—	(0·81)
Carboxyphosphamide¶	—	—	—	(0·40)

Solvent D was run off the leading edge of the chromatograms. R_X values were calculated by reference to the movement of allylmercapturic acid (figures in italics) or cyclophosphamide (figures in parenthesis).

Compound was detected as:

† white spot with Pt reagent;

‡ white spot with the Pt reagent and blue spot with ninhydrin;

§ blue spot with ninhydrin;

¶ blue-mauve spot with the nitrobenzylpyridine reagent.

Gas–liquid chromatography

All g.l.c. separations were carried out on a Perkin–Elmer F-11 gas chromatograph fitted with a dual flame ionization detector and coiled steel columns (2 m × 2·2 mm int. diam.). The column packing material, supplied by Perkin–Elmer Ltd, Beaconsfield, Bucks., UK, was acid-washed silanized chromosorb G (80–100 mesh) coated with either 5% fluorosilicone oil, FS 1265 (column A) or 2·5% silicone gum rubber, E 301 (column B). The carrier gas was nitrogen.

Retention times for trimethylsilyl derivatives of hydroxypropylmercapturic acids are given in table 2.

Infra-red spectrophotometry

The i.r. spectra of synthetic and biosynthetic compounds were determined by the mull method using a Perkin–Elmer 237 infra-red spectrophotometer.

Scintillation counting of ^{14}C

An aq. soln. (0·2 ml) of the radioactive sample was mixed with 10 ml of PCS scintillant (Hopkin and Williams Chemicals Ltd, Romford, Essex) in glass vials and counted in an ICN-Tracerlab Corumatic β-counter. Urine and bile samples were diluted four times with water before counting.

Table 2. Separation of trimethy

Trimethylsilyl derivative in pyridine of:

3-Hydroxypropylmercapturi
2-Hydroxypropylmercaptur
2-Hydroxy-1-methylethylm
An equimolar mixture of th
three derivatives

Retention times were me
sample injection.
† These retention time
derivatives of 2-hydroxyp
acid.

Radioactive compounds on p
papers with water. The aqueous
evaporated to near-dryness in a
reduced pressure. The residue
All samples counted were app

Scanning of chromatograms for
Thin-layer chromatograms
Paper chromatograms were tho
Packard model 7200 radiochrom
ratemeter.

Autoradiography
After thorough drying, pape
seven days.

Degradation of mercapturic acid
Hydroxypropylmercapturic
L-cysteine was degraded to alani
The mercapturic acid (1–5 m
containing 1 mg/ml grade 1 ho
mixture was then applied to a
subsequently washed with wate
resin with ten column volumes
30°C under reduced pressure.
for *S*-(hydroxypropyl)-L-cystei
to dryness and the residue was r
mixture was incubated for 15
supernatant together with wat
examined for alanine by paper

Raney nickel degradation of 3-
Raney nickel quantitatively
1976).
The samples of 3-hydroxy[
degraded with Raney nickel as
supernatant was combined wi
column of Zeo-Karb 225 (H^+
by ten column volumes of 3
evaporated to dryness at 30°C
samples of the soln. were exami

Table 2. Separation of trimethylsilyl derivatives of hydroxypropylmercapturic acids by gas–liquid chromatography.

Trimethylsilyl derivative in pyridine of:	Retention time (min) on column		
	A at 200°C	B at 200°C	B at 180°C
3-Hydroxypropylmercapturic acid	13·1	5·3	10·4
2-Hydroxypropylmercapturic acid	9·6	3·7	7·4
2-Hydroxy-1-methylethylmercapturic acid	9·3	3·6	7·4
An equimolar mixture of the above three derivatives	13·2	5·2	10·4
	9·6†	3·6†	7·4†

Retention times were measured from the start of the pyridine peak and not from the time of sample injection.

† These retention times represent a single peak corresponding to the trimethylsilyl derivatives of 2-hydroxypropylmercapturic acid and 2-hydroxy-1-methylethylmercapturic acid.

Radioactive compounds on paper chromatograms were counted after they had been eluted from the papers with water. The aqueous eluate, or a portion of it, contained in a scintillation counting vial, was evaporated to near-dryness in a current of warm air and then to complete dryness over P_2O_5 under reduced pressure. The residue was counted as above.

All samples counted were approx. equally quenched. The efficiency of counting of ^{14}C was about 90%.

Scanning of chromatograms for ^{14}C

Thin-layer chromatograms were scanned with a Berthold model II radiochromatogram scanner. Paper chromatograms were thoroughly dried, cut into strips (3–5 cm wide) and then scanned with a Packard model 7200 radiochromatogram scanner which was connected to a Packard model 385 recording ratemeter.

Autoradiography

After thorough drying, paper chromatograms were exposed to Kodirex X-ray film in the dark for seven days.

Degradation of mercapturic acids

Hydroxypropylmercapturic acid was deacetylated with acylase and the resultant *S*-(hydroxypropyl)-L-cysteine was degraded to alanine with Raney nickel by a modification of the method of Barnsley (1966).

The mercapturic acid (1–5 mg) was incubated at 37°C for 24 h in 0·1 M sodium phosphate buffer, pH 7, containing 1 mg/ml grade 1 hog kidney acylase (Sigma Chemicals Ltd, Poole Dorset). The incubation mixture was then applied to a column (10 × 1·5 cm diam.) of Zeo-Karb 225 (H^+ form) which was subsequently washed with water until the pH of the eluate was 5. Amino acids were displaced from the resin with ten column volumes of 3 M ammonia and the ammoniacal eluate was evaporated to dryness at 30°C under reduced pressure. The residue was redissolved in water (0·5–1 ml) and the soln. was examined for *S*-(hydroxypropyl)-L-cysteine by paper chromatography. The remainder of the soln. was evaporated to dryness and the residue was redissolved in 0·17 M acetic acid (3 ml). Raney nickel (2 g) was added and the mixture was incubated for 15 h at 50°C in a sealed tube. The mixture was then centrifuged and the supernatant together with water washings of the nickel was treated with Zeo-Karb, as before, and examined for alanine by paper chromatography.

Raney nickel degradation of 3-hydroxy[^{14}C]propylmercapturic acid

Raney nickel quantitatively converts 3-hydroxypropylmercapturic acid into *N*-acetylalanine (Giles 1976).

The samples of 3-hydroxy[^{14}C]propylmercapturic acid was dissolved in 0·125 M acetic acid (4 ml) and degraded with Raney nickel as previously described. The reaction mixture was then centrifuged and the supernatant was combined with several subsequent water washings of the nickel and passed through a column of Zeo-Karb 225 (H^+ form). The column was washed with ten column volumes of water followed by ten column volumes of 3 M ammonia, and the aq. and ammoniacal eluates were combined and evaporated to dryness at 30°C under reduced pressure. The residue was redissolved in water (2 ml) and samples of the soln. were examined by paper chromatography and counted for ^{14}C as described in the text.

Examination of blood samples for mercapturic acids and related compounds

The rat was anaesthetized with diethyl ether and blood (5 ml) was removed from the posterior region of the dorsal aorta using a heparinized plastic syringe. The blood sample was mixed with 10 ml of ice-cold 10 mM phosphate buffer, pH 7·4, and left in ice for 30 min. Protein was pptd. by addition of 5·5% (w/v) $ZnSO_4$ (10 ml) followed by 4·5% (w/v) $Ba(OH)_2$ (10 ml). The mixture was then centrifuged for 20 min at 9000*g* and 4°C in an MSE High Speed 18 centrifuge (8 × 50 ml rotor). The clear supernatants of blood samples from each of six rats were combined and applied to a column (10 × 1·5 cm diam.) of Zeo-Karb 225 (H^+ form). Water was passed through the column until the pH of the eluate was 5, and elution was continued with ten column volumes of 3 M ammonia. The aq. and ammoniacal eluates were separately evaporated to dryness at 30°C under reduced pressure and the residues were each redissolved in water (0·5–1 ml). The solutions were examined by paper chromatography.

Examination of the liver for mercapturic acids and related compounds

The rat was killed by cervical dislocation and the liver was quickly removed and washed in ice-cold water. The liver was transferred to ice-cold water (6 ml/g wet weight of tissue), cut with scissors and homogenized in a mechanical homogenizer fitted with a teflon pestle. Protein was pptd. by addition to the homogenate of 5·5% (w/v) $ZnSO_4$ followed by 4·5% (w/v) $Ba(OH)_2$ (2 ml each soln./g wet wt of liver homogenized). The mixture was centrifuged, treated with Zeo-Karb and examined by paper chromatography as described for the blood samples. Glycogen was removed from the Zeo-Karb aq. eluate concentrate before paper chromatography by addition to the concentrate of ethanol followed by centrifugation.

Cannulation of the bile duct of a rat

The rat (wt. 300 g) was anaesthetized with an i.p. dose (60 mg/kg) of sodium pentobarbital and the abdominal cavity was opened. A plastic cannula was inserted into the bile duct and led out of the rat ventrally into a plastic tube (10 ml capacity) which was strapped to the animal. The incision was stitched and the rat was allowed 3 h to recover before any further experimental work was carried out. On recovery the animal was placed in a restraining cage but had full access to food and water.

Purification of mercapturic acids from urine

Small volumes of urine (below 150 ml) were concentrated to about 50 ml and then adjusted to pH 4 with conc. HCl. Larger volumes of urine were adjusted to pH 2 with conc. HCl and were extracted several times by shaking for 1 h periods with excess butan-2-one (saturated with water). The combined extracts were dried (anhydr. Na_2SO_4) and evaporated to dryness. The residue was redissolved in the minimal volume of 0·1 M sodium formate buffer, pH 4, and the soln. was adjusted, if necessary, to pH 4 with 1 M NaOH.

The concentrated urine sample or butan-2-one soluble material was passed through a column (30 × 3 cm diam.) of Amberlite CG 400 (formate form; 100–200 mesh) which was subsequently eluted with 0·1 M sodium formate buffer, pH 4, until 200 fractions of 10 ml had been collected. Every fifth fraction was concentrated ten-fold and examined for hydroxypropylmercapturic acid by paper chromatography. Fractions containing mercapturic acid were combined and desalted by passage through a column of Zeo-Karb 225 (H^+ form). Water was passed through the column until the eluate had a pH of 5, and then the total eluate was evaporated to dryness at 30°C under reduced pressure. The residue (partially purified mercapturic acid) was redissolved in water (1 ml) and left over moistened NaOH pellets for two days under reduced pressure, and finally dried over P_2O_5 under vacuum.

Isolation of 3-hydroxypropylmercapturic acid as its dicyclohexylammonium salt

A partially purified sample of mercapturic acid was obtained from the urine of rats dosed with cyclophosphamide as described in the previous section. The sample was dissolved in 10 ml of 0·1 M sodium formate buffer, pH 4, and the solution was passed through a column (19 × 1 cm diam.) of Amberlite CG 400 (formate form). The column was washed with water (75 ml) followed by 200 ml of 0·2 M sodium formate buffer, pH 4, and collection of the eluate was started when its pH was 4. The eluate was then desalted by passage through a column of Zeo-Karb 225, as previously described, and finally evaporated to dryness at 30°C under reduced pressure. The residue was redissolved in water (0·5 ml) and stood over moistened NaOH pellets under reduced pressure for three days. The dry (formic acid-free) residue was redissolved in acetone (1 ml) and a 10% (v/v) soln. of dicyclohexylamine in acetone was added until the apparent pH of the soln. (when evaporated on narrow range indicator paper) was 7–8. During storage of the soln. at 5°C for 48 h, a white cryst. ppt. separated. The crystals were washed with cold acetone and then dried over P_2O_5 under reduced pressure before examination by i.r. spectroscopy.

The reaction of acrolein with reduced glutathione in vitro

GSH (310 mg) was mixed with water (1 ml) and the pH was adjusted to 8·5 with 2 M NaOH. Acrolein (73 µl) in ethanol (1 ml) was added and the reaction mixture was left in the light at 20°C for 15 min. Samples (10 µl) were then removed for paper chromatography after which sodium borohydride (0·9 g) was

added, slowly, with continuous sti
times with water, cooled on ice and
through a column (35 × 2 cm diam.
the eluate pH was 5, and then wi
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Results

Identification of 3-hydroxy
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Isolation of 3-hydroxyp
cyclophosphamide

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added, slowly, with continuous stirring. After standing overnight at 20°C, the mixture was diluted five times with water, cooled on ice and adjusted to pH 5 with glacial acetic acid. The resultant soln. was passed through a column (35 × 2 cm diam.) of Zeo-Karb 225 (H^+ form). The column was eluted with water, until the eluate pH was 5, and then with ten column volumes of 3 M ammonia. The ammoniacal eluate was evaporated to dryness, and the residue was redissolved in water (2 ml) and examined by paper chromatography. The major product present was subsequently purified by preparative paper chromatography in solvent B.

Results

Identification of 3-hydroxypropylmercapturic acid in the urine of rats dosed with cyclophosphamide, isophosphamide and trilophosphamide

When cyclophosphamide, isophosphamide or trilophosphamide were administered to rats, the urines excreted during the first 24 h after dosage contained a compound, sensitive to the Pt reagent for sulphur compounds, which was identical to synthetic 3-hydroxypropylmercapturic acid on paper chromatography in solvents A, B, C and D. This compound was absent from the urine of control rats dosed with saline or arachis oil and was not detectable in urine excreted by rats during the second 24 h period after administration of drugs. Excretion of the compound was independent of the route of administration (s.c., i.p., or gavage) of cyclophosphamide. Moreover, the behaviour of the compound on chemical degradation was characteristic of a mercapturic acid (Barnsley 1966) and was identical to synthetic 3-hydroxypropylmercapturic acid. Thus deacetylation with acylase yielded a product, sensitive to the Pt reagent and ninhydrin, which was chromatographically similar to synthetic 3-hydroxypropylcysteine, and Raney nickel degradation of the deacetylation product yielded alanine.

When partially purified samples of the mercapturic acid were prepared from the urine of 30, 12 and 60 rats dosed with cyclophosphamide (s.c.), isophosphamide and trilophosphamide respectively, g.l.c. of the silylated samples demonstrated, in each case, a peak with the same retention times as synthetic trimethylsilyl 3-hydroxypropylmercapturate on both columns A and B. Urine was subsequently collected for 48 h from 30 rats dosed with saline or arachis oil and dicyclohexylammonium 2-hydroxypropylmercapturate (150 mg) was added. When the mercapturic acid was partially purified from the urine, g.l.c. of the silylated material gave a single peak corresponding to trimethylsilyl 2-hydroxypropylmercapturate, but no peaks with the same retention times as trimethylsilyl 3-hydroxypropylmercapturate indicating the absence of the latter mercapturic acid (at detectable levels) in the urine of the control animals (the isomers 2-hydroxy- and 3-hydroxy-propylmercapturic acids do not separate on Amberlite CG 400 fractionation of urine samples and extracts as described in the methods).

Isolation of 3-hydroxypropylmercapturic acid from the urine of rats dosed with cyclophosphamide

After g.l.c. of the trimethylsilyl derivative in pyridine, as described in the previous section, the remainder of the sample of the mercapturic acid, partially purified from the urine of rats dosed with cyclophosphamide, was evaporated to dryness. The residue was redissolved in sodium formate buffer and the mercapturic acid was isolated as its dicyclohexylammonium salt. After drying, the salt (5·5 mg) had an uncorrected m.p. (decomp.) of 172–175°C and its i.r. spectrum (figure 3) was identical to that of synthetic dicyclohexylammonium 3-hydroxypropylmercapturate (uncorrected m.p. [decomp.], 173°C).

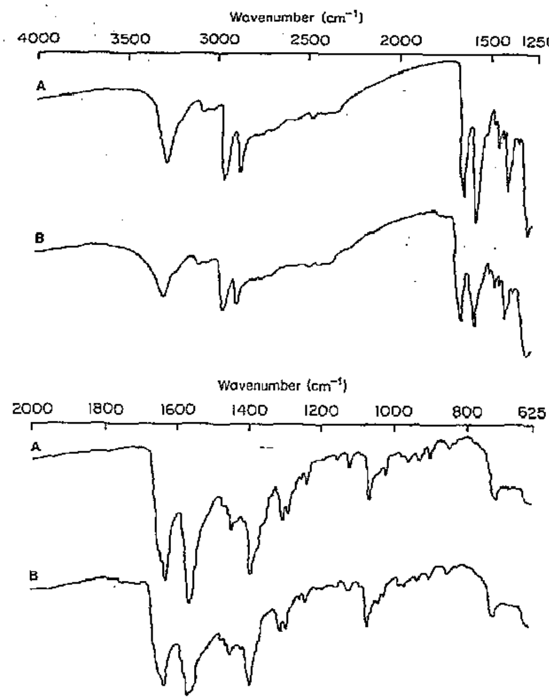

Figure 3. Infra-red spectra of synthetic and biosynthetic 3-hydroxypropylmercapturic acid. A: Synthetic dicyclohexylammonium 3-hydroxypropylmercapturate. B: Dicyclohexylammonium salt of the mercapturic acid isolated from the urine of rats dosed with cyclophosphamide.

Excretion in the urine of radioactive metabolites of [^{14}C]cyclophosphamide

Scintillation counting of urine, collected for 48 h from three rats dosed with [^{14}C]cyclophosphamide, showed that of the total administered dose of ^{14}C, 55·5% was excreted during the first 24 h period after dosage and a further 6·6% was excreted in the subsequent 24 h.

Samples of the first 24 h urine were subjected to paper chromatography in solvent D for 7 h and 26 h, and the chromatograms were scanned for radioactivity and exposed to X-ray film (figure 4). ^{14}C-Labelled compounds with identical R_X values to synthetic 3-hydroxypropylmercapturic acid, carboxyphosphamide and cyclophosphamide were identified, and from estimation of the peak areas on the scanner ratemeter recordings, these represented approx. 13·5%, 41·6% and 19·3% respectively of the total urinary radioactivity. The value for cyclophosphamide may, however, be high, as the peak corresponding to this compound was incompletely resolved from a second peak due to an alkylating metabolite, although examination of the autoradiograph showed that these two compounds had just separated. The major metabolite was carboxyphosphamide. Cyclophosphamide and carboxyphosphamide were visualized on the chromatograms with the nitrobenzylpyridine reagent, and 3-hydroxypropylmercapturic acid was detected with the Pt reagent. No attempt was made to identify any other metabolites, of which there were at least six indicated on the autoradiograph.

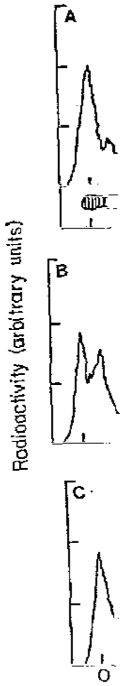

Figure 4. Paper chromatogram
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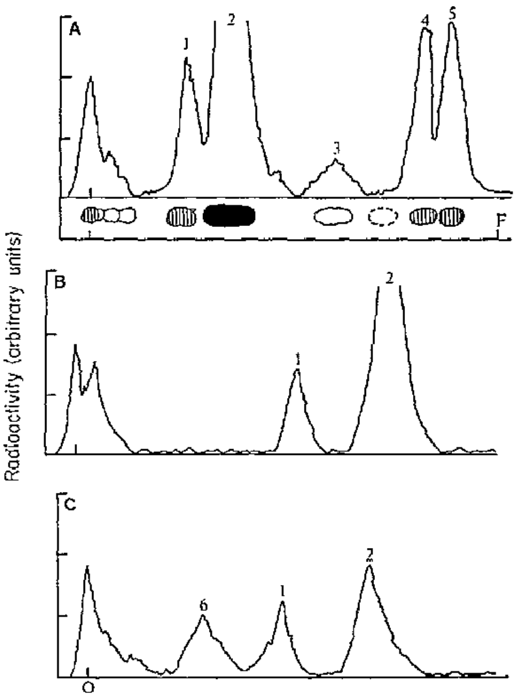

Figure 4. Paper chromatogram scans of urine and bile from rats dosed with $[^{14}C]$cyclophosphamide. Ratemeter recordings from scanning of paper chromatograms for ^{14}C are drawn to scale. Samples of first 24-hour urine from rats dosed with $[^{14}C]$cyclophosphamide were separated by paper chromatography in solvent D for 7 h (A) and 26 h (B). Some of the peaks, recorded from scanning of A, were incompletely separated, but the autoradiograph of the same chromatogram (shown diagrammatically below A) showed that the radioactive compounds corresponding to these peaks were just resolved. Bile samples from a rat dosed with $[^{14}C]$cyclophosphamide were subjected to paper chromatography in solvent D for 22 h (C). Solvent D ran off the leading edge of the paper during 22 h development of chromatograms. O, Origin; 1,3-hydroxypropylmercapturic acid; 2, carboxyphosphamide; 3 and 4, alkylating agents; 5, cyclophosphamide; 6, *S*-(-hydroxypropyl)-L-cysteine; F, solvent front. Degree of development of autoradiograph film: solid areas, very strong; hatched areas, medium to strong; clear areas, weak; dotted area, very faint.

A comparison of the relative conversion of $[^{14}C]$cyclophosphamide into 3-hydroxy$[^{14}C]$propylmercapturic acid and $[^{14}C]$carboxyphosphamide during the first 24 h after drug administration was made by scintillation counting of the metabolites purified from the urine (1·5 ml) by preparative paper chromatography in solvent D for 26 h. Paper chromatography of aq. solutions (1 ml) of the purified metabolites on Whatman no. 1 papers in solvents A, B, C and E showed, in the case of each metabolite, a single radioactive compound when the papers were scanned for ^{14}C. Both of the purified compounds ran identically to the respective synthetic compounds in all four solvent systems and were accordingly visualized as single spots with the Pt reagent (3-hydroxypropylmercapturic acid) and the nitrobenzyl-pyridine reagent (carboxyphosphamide). Scintillation counting of the solutions of the chromatographically pure metabolites gave values of 41·6% and 11·9% for the percentage of the total ^{14}C in the first 24 h urine represented respectively as carboxyphosphamide and 3-hydroxypropylmercapturic acid.

Localization of the ^{14}C label in 3-hydroxypropylmercapturic acid in the urine of rats dosed with [^{14}C]cyclophosphamide

A radiochromatographically pure sample of 3-hydroxy[^{14}C]propylmercapturic acid was prepared by preparative paper chromatography in solvent D (for 26 h) from urine (6 ml) excreted by three rats during the first 24 h after administration of [^{14}C]cyclophosphamide.

The radioactivity of the purified mercapturic acid was assessed by scintillation counting, after which it was degraded with Raney nickel. Scintillation counting of the Raney nickel degradation mixture, after Zeo-Karb treatment, demonstrated the loss of 86·4% of the original radioactivity present in the 3-hydroxy[^{14}C]-propylmercapturic acid. When samples of the Zeo-Karb-treated degradation mixture were subjected to paper chromatography in solvent C, and the area of each chromatogram approximating to *N*-acetylalanine (detected by standards at the edge of the paper) was eluted with water, scintillation counting of the eluates indicated that this area contained only 2·2% of the ^{14}C present in the original radiochromatographically pure sample of 3-hydroxy[^{14}C]propylmercapturic acid. The recovery, by the method described, of non-radioactive samples of *N*-acetylalanine from Raney nickel degradation mixtures was always >98%, as determined by titration of this compound with NaOH.

The results demonstrate that the ^{14}C label in the 3-hydroxypropylmercapturic acid excreted in the urine of rats dosed with [^{14}C]cyclophosphamide is located in the *S*-substituent and not in the *N*-acetylcysteine moiety.

Paper chromatographic examination of the bile of a rat dosed with [^{14}C]-cyclophosphamide

Bile was collected from a rat for 5 h after an i.p. dose (120 mg/kg) of [^{14}C]cyclophosphamide (sp. activity 0·195 μCi/mg). The bile was concentrated to 1 ml at 30°C under reduced pressure and scintillation counting of the concentrate showed that it contained 1·9% of the total administered dose of ^{14}C. Separation of samples of the concentrate by paper chromatography in solvent D for 26 h and subsequent scanning of the papers for radioactivity indicated the presence of ^{14}C-labelled compounds with the same R_X values (where X was carboxyphosphamide) as carboxyphosphamide, 3-hydroxypropylmercapturic acid and 3-hydroxypropylcysteine (figure 4). The latter two compounds were detected in trace amounts with the Pt reagent, and carboxyphosphamide was visualized with the nitrobenzylpyridine reagent. When samples of the bile concentrate were separated by paper chromatography in solvents A and B, scanning for ^{14}C and treatment of the papers with the Pt and dinitrophenylhydrazine reagents did not show any 3-hydroxypropylglutathione or aldehydes.

The ^{14}C-labelled compound which had an identical R_X value to synthetic 3-hydroxypropylcysteine was purified from the remainder of the bile concentrate by preparative paper chromatography in solvent D for 26 h. Paper chromatography of the purified compound in solvents A, B and C showed a single radioactive compound with the same R_F values as synthetic 3-hydroxypropylcysteine. These results indicate the formation of 3-hydroxypropylcysteine as a metabolite of [^{14}C]cyclophosphamide in the rat.

Examination of the liver of r
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Examination of the liver of rats after administration of cyclophosphamide, allyl alcohol and acrolein

Liver was removed from rats 20 min and 40 min after administration of acrolein or allyl alcohol, and 40 min and 80 min after i.p. administration of cyclophosphamide (500 mg/kg). The rats dosed with cyclophosphamide had been pretreated with phenobarbital. Paper chromatography of liver extracts showed traces of 3-hydroxypropylmercapturic acid 80 min and 40 min after dosage with cyclophosphamide and acrolein respectively, but 3-hydroxypropylmercapturic acid and 3-hydroxypropylcysteine were detectable 40 min after administration of allyl alcohol. Treatment of the chromatograms with the Pt and dinitrophenylhydrazine reagents did not demonstrate any other sulphur-containing or aldehydic metabolites of cyclophosphamide, allyl alcohol or acrolein. No evidence was obtained for the presence of 3-hydroxypropylmercapturic acid or 3-hydroxypropylcysteine in the liver of control rats dosed with arachis oil or saline.

Examination of the blood of rats dosed with cyclophosphamide

Cyclophosphamide (400 mg/kg) was administered intraperitoneally to 18 rats and blood samples were removed and pooled from six rats at 0·5, 1 and 2 h after dosage. Paper chromatography indicated traces of 3-hydroxypropylmercapturic acid in the 1 and 2 h samples. No other sulphur-containing metabolites of cyclophosphamide were found. 3-Hydroxypropylmercapturic acid was not present in blood samples taken from control rats dosed with saline.

Reaction of acrolein with GSH in vitro

Paper chromatography of the reaction mixture in solvent A resolved two dinitrophenylhydrazine-sensitive compounds (R_F 0·10 and 0·28) which were presumed to be aldehydes. After reduction with borohydride, paper chromatography of the reaction mixture in solvent B demonstrated one major compound and at least five minor compounds which were visualized with ninhydrin and/or the Pt reagent. The major compound, after purification by preparative paper chromatography in solvent B, had identical R_F values to synthetic 3-hydroxypropylglutathione on paper chromatography in solvents A, B and C and was detected as a single spot with ninhydrin and the Pt reagent.

Metabolism of the reduced major product of the reaction of acrolein with GSH in vitro

The remainder of the purified reduced major product, as described in the preceding section, was dissolved in 0·9% (w/v) NaCl (1 ml) and administered (i.p.) to a rat. Paper chromatographic examination of the urine collected during the subsequent 24 h showed one major and two minor sulphur-containing (Pt reagent sensitive) metabolites. In solvents A, B and C the major metabolite had the same R_F values as synthetic 3-hydroxypropylmercapturic acid, and the two minor metabolites were chromatographically identical to metabolites II and III of 3-hydroxypropylglutathione (table 3).

*Metabolism of S-(3-hydroxypropyl)glutathione, S-(3-hydroxypropyl)-*L*-cysteine and 3-hydroxypropylmercapturic acid* in vivo

Rats were dosed with 3-hydroxypropylglutathione, 3-hydroxypropylcysteine or saline and the urine was collected for 24 h or the liver was removed after 40 min. Samples of urine and extracts of the liver were examined by paper chromatography in solvents A, B, C and D.

Table 3. Paper chromatographic examination of sulphur-containing metabolites of *S*-(3-hydroxypropyl)glutathione, *S*-(3-hydroxypropyl)-L-cysteine and 3-hydroxypropylmercapturic acid in urine samples.

Synthetic compounds:	R_F in solvent A	R_F in solvent B	R_F in solvent C	R_X in solvent D
3-Hydroxypropylmercapturic acid	0·77	0·55	0·50	0·52
3-Hydroxypropylmercapturic acid sulphoxide†	0·49	0·39	0·40	n.d.
S-(3-Hydroxypropyl)-L-cysteine-*S*-oxide†§	0·40	0·40	0·37	0·21
2-Carboxyethylmercapturic acid	0·77	0·27	0·21	0·15
2-Carboxyethylmercapturic acid sulphoxide†	0·47	n.d.	0·13	n.d.
Metabolites of:				
S-(3-Hydroxypropyl)glutathione				
(Metabolite I)	0·76	0·55	0·50	0·53
(Metabolite II)†	0·36	0·37	0·33	0·25
(Metabolite III)	0·72¶	0·22‡	0·16‡	0·14‡
S-(3-Hydroxypropyl)-L-cysteine				
(Metabolite I)	0·77	0·55	0·50	0·53
(Metabolite II)†	0·36	0·37	0·33	0·26
(Metabolite III)	0·73¶	0·23‡	0·16‡	0·14‡
3-Hydroxypropylmercapturic acid				
(Metabolite IV)†	0·36	0·40	0·37	0·30
(Metabolite V)	0·77¶	0·26‡	0·22‡	0·15‡

All compounds were detected with the Pt reagent.

† Compound gave a yellow spot, characteristic of sulphoxides, when the chromatogram was dipped in the Pt reagent and then heated in a stream of warm air.

‡ R_F value obtained for both the purified metabolite (prepared as below) and the metabolite in the urine.

§ Compound visualized as a blue spot with ninhydrin.

¶ Metabolite was unresolved from 3-hydroxypropylmercapturic acid in solvent A. The R_F value was obtained after purification of the metabolite from the urine by preparative paper chromatography in solvent C.

n.d.: R_F or R_X value not determined.

All R_X values were obtained by reference to the movement of allylmercapturic acid. The R_F and R_X values of all metabolites were obtained from paper chromatography of untreated urine samples and also from urine (pH 6·5) which had been desalted by passage through a column of Zeo-Karb 225 (H^+ form). Desalting of the urine did not alter the chromatographic behaviour of the metabolites. All of the metabolites were neutral or negatively charged at pH 6·5 as none of them exchanged on to the Zeo-Karb.

3-Hydroxypropylcysteine and 3-hydroxypropylmercapturic acid were detected in the liver of rats dosed with 3-hydroxypropylglutathione and 3-hydroxypropylcysteine, but not in liver removed from animals dosed with saline. 3-Hydroxypropylglutathione was not detectable in the liver after administration of either of these compounds.

Examination of the urine showed that rats dosed with 3-hydroxypropylglutathione excreted three sulphur-containing metabolites of this compound which were chromatographically identical to three metabolites excreted in the urine of animals administered 3-hydroxypropylcysteine and which were absent from the urine of control rats dosed with saline (table 3). All three metabolites of 3-hydroxypropylglutathione and 3-hydroxypropylcysteine were visualized with the Pt reagent but were insensitive to ninhydrin. Metabolite I, the major metabolite of each compound, had R_F values identical to synthetic 3-hydroxypropylmercapturic acid. Moreover, when metabolite I from 3-hydroxypropylglutathione was partially purified from the urine of ten rats, g.l.c. of the silylated material confirmed that the

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Discussion

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metabolite was 3-hydroxypropylmercapturic acid. Metabolites II and III were not identified.

When the urine of rats dosed with 3-hydroxypropylmercapturic acid was separated by paper chromatography, a large amount of the unchanged mercapturic acid was found together with small amounts of two metabolites which were undetectable in the urine of rats dosed with saline. The metabolites were sensitive to the Pt reagent but not to ninhydrin, and they migrated slightly differently to the urinary metabolites of 3-hydroxypropylglutathione and 3-hydroxypropylcysteine (table 3). Metabolite IV was not identified, but metabolite V had the same R_F and R_X values as synthetic 2-carboxyethylmercapturic acid and was therefore tentatively identified as this compound.

None of the metabolites of 3-hydroxypropylmercapturic acid or metabolites II and III of 3-hydroxypropylglutathione and 3-hydroxypropylcysteine were detected in the urine of rats dosed with cyclophosphamide.

Discussion

The isolation of 3-hydroxypropylmercapturic acid, as its dicyclohexylammonium salt, from the urine of rats dosed with cyclophosphamide appears to be the first reported isolation of the mercapturic acid from the urine of animals dosed with this drug. Moreover, the detection of 3-hydroxy[^{14}C]propylmercapturic acid in the urine of rats dosed with [^{14}C]cyclophosphamide confirms that the mercapturic acid is a metabolite of the drug. The observations that the cyclophosphamide analogues isophosphamide and trilophosphamide are also converted into 3-hydroxypropylmercapturic acid by the rat (Giles 1976, Alarcon 1976) indicate some similarity in the metabolism of the two analogues and the parent drug *in vivo*.

A comparison of the relative conversion of a dose (200 mg/kg) of [^{14}C]cyclophosphamide into carboxyphosphamide and 3-hydroxypropylmercapturic acid during the first 24 h after drug administration showed that these two metabolites represented 23·1% and 6·6% respectively of the administered ^{14}C (41·6% and 11·9% respectively of the total urinary ^{14}C). The excretion in the urine of carboxyphosphamide as the major metabolite of cyclophosphamide in rats is in agreement with the results of previous studies on the metabolism of cyclophosphamide in animals (Struck *et al.* 1971, Takamizawa *et al.* 1972, Bakke *et al.* 1972) and humans (Struck *et al.* 1971). The amount of [^{14}C]cyclophosphamide converted by rats into 3-hydroxypropylmercapturic acid was lower than the 9·7% conversion of non-radioactive cyclophosphamide (14–84 mg/kg) into the mercapturic acid obtained in a previous study (Alarcon 1976). This might, however, be explained by the fact that much higher doses of cyclophosphamide were administered to animals in the present work.

An examination of the structure of cyclophosphamide (figure 1) would indicate that the three-carbon unit of the oxazaphosphorine ring is most likely to be incorporated into the *S*-substituent of 3-hydroxypropylmercapturic acid *in vivo*, and consistent with this is the finding that the mercapturic acid, excreted by rats in the first 24 h after a single dose of [^{14}C]cyclophosphamide (200 mg/kg), is significantly labelled only in the *S*-substituent. Some previous reports, such as those describing the identification of *O*-2-hydroxyethyl-*N*,*N*-bis(2-chloroethyl)phosphodiamidate as a metabolite of cyclophosphamide in sheep urine (Bakke *et al.* 1972) and the detection of $^{14}CO_2$ in the expiratory air of mice dosed with ring ^{14}C-labelled cyclophosphamide (Rutman 1964), indicate that the cyclophosphamide ring can

undergo carbon–carbon bond scission in certain systems. Whether repeated doses of [^{14}C]cyclophosphamide over a long period would lead to any labelling of the N-acetylcysteine moiety of 3-hydroxypropylmercapturic acid was not determined in the present work.

The formation of 3-hydroxypropylmercapturic acid from cyclophosphamide *in vivo* must involve the reaction of one or more cyclophosphamide metabolites with a thiol, and the metabolite in question is currently thought to be acrolein (Kaye and Young 1974). The interaction of the kryptaldehyde group of 4-hydroxycyclophosphamide (figure 1) with thiols has been demonstrated, but this results in the formation of 4-(S-R)mercaptocyclophosphamide derivatives and not in the formation of compounds with *S*-3-hydroxypropyl groups (Peter, Wagner and Hohorst 1976, Wagner *et al.* 1977). The α, β double bond of acrolein is strongly polarized by the aldehydic oxygen and it is consequently highly reactive towards cell nucleophiles (-OH, -NH_2, -SH), especially -SH groups (Munsch, Recondo and Frayssinet 1974). The occurrence of GSH as the predominant source of cellular non-protein thiol (Beutler, Duron and Kelly 1963, Koivusala and Uotila 1974, Crowley, Gillham and Thorn 1975) makes this compound a prime target for reaction with any acrolein released from cyclophosphamide *in vivo*. Although an examination, in the present study, of the liver, bile, blood and urine of rats dosed with cyclophosphamide, and the liver of rats dosed with allyl alcohol and acrolein did not demonstrate any glutathione conjugates, the detection of 3-hydroxypropylmercapturic acid in the blood of rats only 60 min after administration of cyclophosphamide is consistent with the interaction of a cyclophosphamide metabolite with a thiol, such as GSH, which has a relatively short biological half-life.

Theoretically, acrolein would be expected to react with GSH yielding *S*- and *N*-2-aldehydo-ethyl derivatives. The detection of 3-hydroxypropylglutathione as the major product in the acrolein–GSH reaction mixture after reduction with borohydride indicates the formation of *S*-(2-aldehydo-ethyl)glutathione as the major product of the reaction of acrolein with GSH. 2-Aldehydo-ethylglutathione was therefore probably one of the two aldehydes detected in the reaction mixture before reduction. However, the detection of at least six products in the borohydride-reduced reaction mixture demonstrates the complexity of the reaction of acrolein with GSH *in vitro* which may not be the case *in vivo*. If acrolein is formed from cyclophosphamide *in vivo*, the slow release and consequent low tissue concentration of acrolein might favour the formation of 2-aldehydo-ethylglutathione as a product of its reaction with GSH, especially if the reaction was enzyme-catalysed (figure 5).

Studies on the metabolism of acrolein *in vivo* are hampered by the high reactivity of the compound. Hence examination of the liver of rats dosed subcutaneously with acrolein demonstrated traces of 3-hydroxypropylmercapturic acid but no mercapturic acid precursors, probably because none of the administered acrolein had reached the liver. Furthermore, Kaye (1971, 1973) recovered only 10·3% of a subcutaneous dose of acrolein as 3-hydroxypropylmercapturic acid from the urine of rats. In the current study, allyl alcohol was consequently used as an *in vivo* precursor of acrolein in the liver in an attempt to mimic the possible formation of the latter compound from cyclophosphamide. The validity of using allyl alcohol in this way was based on the previous observations that rat liver cannot directly conjugate allyl alcohol with GSH *in vitro* (Kaye 1971), allyl alcohol is converted into acrolein in the liver by an NAD$^+$-dependent dehydrogenase (Serafini Cessi 1972), and that allyl alcohol is metabolized, probably via the formation of acrolein (Kaye 1971, 1973),

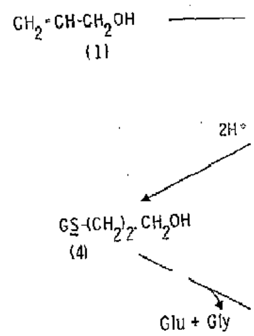

Figure 5. Possible pathways for hydroxypropylmercapturic ethyl)glutathione; (4), S-(glycine; (6), S-(3-hydroxyp (3-hydroxypropyl)-L-cystei propylmercapturic acid. Re (*), during (†) or after (‡) i

into 3-hydroxypropylmerc propylcysteine and 3-hydr with allyl alcohol and in the that the last step in the m from allyl alcohol and cycl cysteine (figure 5). Theref phamide *in vivo*, reacts necessary reduction of the latter compound into 3-hy or after hydrolysis of the acetylation of the resultar predict more precisely the aldehydes were detected a the metabolism of 2-aldeh experienced in preparing metabolism of its reducti

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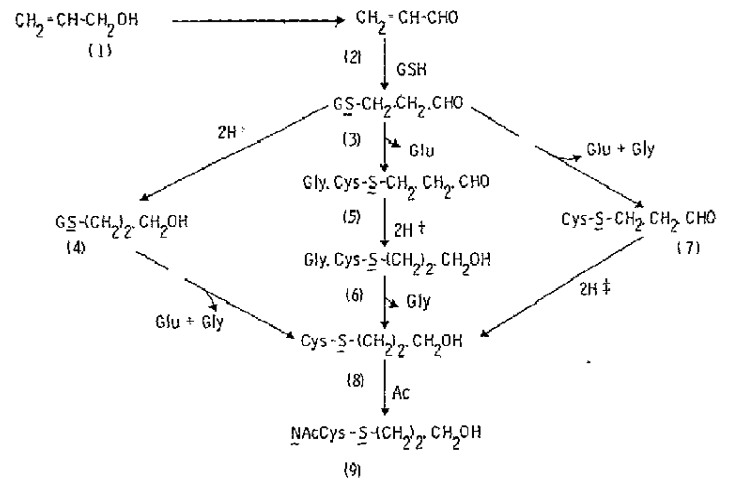

Figure 5. Possible pathways for the metabolic conversion of allyl alcohol and acrolein into 3-hydroxypropylmercapturic acid by the rat. (1), Allyl alcohol; (2), acrolein; (3), *S*-(2-aldehydo-ethyl)glutathione; (4), *S*-(3-hydroxypropyl)glutathione; (5), *S*-(2-aldehydo-ethyl)cysteinyl-glycine; (6), *S*-(3-hydroxypropyl)cysteinylglycine; (7), *S*-(2-aldehydo-ethyl)-L-cysteine; (8), *S*-(3-hydroxypropyl)-L-cysteine; (9), *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine, i.e. 3-hydroxy-propylmercapturic acid. Reduction of *S*-(2-aldehydo-ethyl)glutathione (2*H*) could occur before (*), during (†) or after (‡) its hydrolysis. Ac = Acetyl.

into 3-hydroxypropylmercapturic acid by the rat. The detection of 3-hydroxy-propylcysteine and 3-hydroxypropylmercapturic acid in the liver of rats dosed with allyl alcohol and in the bile of a rat dosed with [^{14}C]cyclophosphamide indicates that the last step in the metabolic formation of 3-hydroxypropylmercapturic acid from allyl alcohol and cyclophosphamide is the *N*-acetylation of 3-hydroxypropyl-cysteine (figure 5). Therefore if acrolein, released from allyl alcohol and cyclophosphamide *in vivo*, reacts with GSH forming 2-aldehydo-ethylglutathione, the necessary reduction of the aldehyde group which occurs during the conversion of the latter compound into 3-hydroxypropylmercapturic acid could occur before, during or after hydrolysis of the glutathione conjugate, but must occur before the *N*-acetylation of the resultant *S*-substituted cysteine (figure 5). It is not possible to predict more precisely the point of reduction, because no glutathione conjugates or aldehydes were detected as metabolites of allyl alcohol or cyclophosphamide. Also the metabolism of 2-aldehydo-ethylglutathione was not studied because of difficulty experienced in preparing suitably pure samples of this compound. However, the metabolism of its reduction product, 3-hydroxypropylglutathione, was studied.

The conversion of *S*-substituted glutathiones (Stekol 1940, 1941, Roberts and Warwick 1958, Foxwell and Young 1964, Gray and Barnsley 1971, Kaye 1971, 1973) and *S*-substituted cysteines (Stekol 1938, Thomson, Barnsley and Young 1963), including 3-hydroxypropylcysteine (Kaye 1971, 1973), into the corresponding mercapturic acid is well documented. The detection in the current work of 3-hydroxypropylcysteine and 3-hydroxypropylmercapturic acid in the liver, and the latter compound in the urine of rats dosed with 3-hydroxypropylglutathione and 3-hydroxypropylcysteine is also consistent with the hypothesis (Bray *et al.* 1959, Barnes *et al.* 1959) that *S*-substituted glutathione is metabolized into mercapturic acid through the intermediate formation of the corresponding *S*-substituted cysteine.

Also the absence of detectable levels of unchanged 3-hydroxypropylglutathione and the detection of 3-hydroxypropylcysteine as the first apparent metabolic product of 3-hydroxypropylglutathione in the liver of rats dosed with the glutathione conjugate indicates that the conjugate is metabolized very quickly by the liver into 3-hydroxypropylcysteine. It is therefore rather unlikely that any glutathione conjugates formed from cyclophosphamide and allyl alcohol *in vivo* would be present in the liver at any one time in significantly detectable amounts, especially as these two compounds would require metabolic transformation before reacting with GSH. This might explain why no conjugates were found in the liver of rats dosed with cyclophosphamide and allyl alcohol, or in the bile of a rat administered [^{14}C]cyclophosphamide.

As the two minor urinary metabolites (II and III) of 3-hydroxypropylglutathione are chromatographically identical to those of 3-hydroxypropylcysteine, but apparently different to the metabolites (IV and V) of 3-hydroxypropylmercapturic acid, it seems likely that metabolites II and III of the first two compounds are derived from 3-hydroxypropylcysteine without the intermediate formation of 3-hydroxypropylmercapturic acid, and that metabolites IV and V of 3-hydroxypropylmercapturic acid are formed directly from the mercapturic acid without the intermediate formation of 3-hydroxypropylcysteine. The conversion of 3-hydroxypropylmercapturic acid into 2-carboxyethylmercapturic acid (metabolite V) *in vivo* possibly involves the initial NAD$^+$-dependent oxidation of the mercapturic acid to *N*-acetyl-*S*-(2-aldehydo-ethyl)-L-cysteine by an alcohol dehydrogenase followed by conversion of the aldehyde into 2-carboxyethylmercapturic acid by aldehyde dehydrogenase (figure 6). Moreover, the NAD$^+$-dependent oxidation of 3-hydroxy-1-methylpropylmercapturic acid (Gray and Barnsley 1971) and 3-hydroxypropylmercapturic acid (P. Callaghan, personal communication) *in vitro* by rat

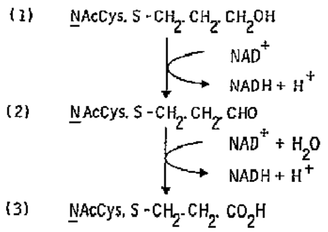

Figure 6. Possible pathway for the conversion of 3-hydroxypropylmercapturic acid into 2-carboxyethylmercapturic acid *in vivo*. (1), 3-Hydroxypropylmercapturic acid; (2), 2-aldehydo-ethylmercapturic acid; (3), 2-carboxyethylmercapturic acid.

liver enzymes has been observed, and if the K_m for the latter reaction is similar to that reported for the former (13 mM), it could explain why no 2-carboxyethylmercapturic acid is excreted in the urine of rats given high doses (200 mg/kg) of cyclophosphamide; the tissue concentration of 3-hydroxypropylmercapturic acid formed from cyclophosphamide *in vivo* would not be high enough to result in any significant oxidation of 3-hydroxypropylmercapturic acid to 2-carboxyethylmercapturic acid. A similar argument could apply to any glutathione conjugates and 3-hydroxypropylcysteine formated from cyclophosphamide *in vivo*, because no evidence was found for the excretion of the minor urinary metabolites (II and III) of 3-hydroxypropylglutathione and 3-hydroxypropylcysteine in the urine of rats dosed

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with cyclophosphamide. In support of this argument are the current observations that the metabolites II and III of 3-hydroxypropylglutathione and 3-hydroxypropylcysteine, and metabolites (IV and V) of 3-hydroxypropylmercapturic acid, are only formed in small amounts, and that when 3-hydroxypropylmercapturic acid is administered to rats it is excreted mainly as the unchanged compound.

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† Present address: Children's
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